Evolutionary responses of energy metabolism, development, and reproduction to artificial selection for increasing heat tolerance in *Drosophila subobscura*.

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Abstract

Adaptations to warming conditions exhibited by ectotherms include increasing heat tolerance but also metabolic changes to reduce maintenance costs (metabolic depression), which can allow them to redistribute the energy surplus to biological functions close to fitness. Currently, there is evidence that energy metabolism evolves in response to warming conditions but we know little about how the rate of temperature change during heat stress determines the evolutionary response of metabolism and the consequences on life-history traits. Here, we evaluated the evolutionary response of energy metabolism (metabolic rate and activity of enzymes of the glucose-6-phosphate branchpoint) and life-history traits to artificial selection for increasing heat tolerance in Drosophila subobscura, using two different thermal selection protocols for heat tolerance: slow and fast ramping protocols. We found that the increase in heat thermal tolerance was associated with a reduction of the hexokinase activity in the slow-ramping selected lines, and a slight reduction of the glucose-6-phosphate dehydrogenase activity in the fast-ramping selected lines. We also found that the evolution of increased heat tolerance increased the early fecundity in selected lines and increased the egg-to-adult viability only in the slow-ramping selected lines. However, heat tolerance evolution was not associated with changes in the metabolic rate in selected populations. This work shows heat tolerance can evolve under different thermal scenarios but with different evolutionary outcomes on associated traits depending on the intensity of thermal stress. Therefore, spatial and temporal variability of thermal stress intensity should be taken into account to understand and predict the adaptive response to ongoing and future climatic conditions.

Keywords: heat tolerance evolution, thermal stress, metabolic rate, G6P branchpoint, early fecundity, preadult viability.

Introduction

Environmental thermal stress involves strong selective pressures that can lead to important evolutionary changes depending on genetic variation exhibited by natural populations (Umina et al., 2005; Crozier & Hutchings, 2014; Chick et al., 2021). The increasing environmental temperatures cause fitness declines and consequently changes in abundance and distribution of ectotherm populations (Deutsch et al., 2008; Angilletta, 2009; Chen et al., 2011). Therefore, knowing and exploring how ectotherms will withstand global warming is crucial.

The ability to tolerate high temperatures implies that organisms must exhibit an array of responses at different levels of organization, allowing them to maintain their biological performance under these conditions (Huey & Kingsolver, 1993; Pörtner et al. 2006; Mesas et al. 2021; McGaughran et al. 2021). Among these responses, metabolic depression, defined as a reduction of the energy expenditure associated with the maintenance of organisms, is considered an important physiological mechanism related to resistance to environmental stress (Guppy & Withers, 1999; Storey & Storey, 2004). Plastic and evolutionary changes in energy metabolism should have important ecological and evolutionary consequences when organisms are exposed to stressful conditions because it should allow them to reduce their minimum energetic requirements and redistribute the energy surplus to biological functions close to fitness (Atkinson, 1977; Storey & Storey, 2004). For example, individuals with a low standard metabolic rate (SMR) have higher survival compared to those individuals with high SMR (Artacho & Nespolo, 2009). Additionally, the evolution of increased resistance to desiccation,

65 starvation, hypoxia, and high temperatures has been also accompanied by a reduction in
66 metabolic rate (Hoffmann & Parson, 1989; Padfield et al. 2016; Regan et al. 2017; Brown et al.
67 2019; but see Djawdan et al. 1997), suggesting that energy-saving phenotypes are favored under
68 stressful conditions.

69 On a broader scale, metabolic depression as a physiological response to high temperature
70 could explain the countergradient variation in metabolic rate proposed in the Metabolic Cold
71 Adaptation hypothesis (Addo-Bediako, 2002; Gaston et al. 2009), which establishes that
72 ectothermic species from temperate habitats have lower metabolic rates than those living in
73 colder environments (Addo-Bediako et al. 2002; Sylvestre et al. 2007; Schaefer & Walters 2010;
74 Sinnatamby et al. 2015; Pilakouta et al. 2020). Also, metabolic depression has represented an
75 adaptive response in phytoplankton populations, which evolved in warm environmental
76 conditions and showed an increase in their thermal tolerance accompanied by a reduced
77 metabolic rate (Padfield et al. 2016). However, there is also evidence that *Drosophila simulans*
78 showed an increase in metabolic rate after 60 generations of evolution in a hot environment
79 (Mallard et al. 2018). Regardless of the direction of the metabolic response to warm conditions,
80 these results indicate that metabolism can evolve when populations are exposed to stable warm
81 conditions. However, under natural conditions, organisms are exposed to fluctuating
82 environmental conditions, which can result in different evolutionary outcomes (Colinet et al
83 2015). For instance, Mesas et al (2021) found an evolutionary increase in heat tolerance in *D.*
84 *subobscura* regardless of the ramping rate used during thermal assays, but changes in the thermal
85 performance curve of the evolved populations did depend on the ramping rates used during the
86 selection. This evidence suggests that heat tolerance may evolve in response to global warming,
87 but responses of associated traits, including metabolic rate, would depend on the rate of

88 temperature change during heat stress (Santos et al 2012). Indeed, individuals with slow
89 metabolism are expected to have a higher heat tolerance than individuals with fast metabolism
90 because the latter depletes energy resources faster than the former during heat stress (Rezende et
91 al. 2011; Santos et al. 2012).

92 Metabolism is an emergent property of interactions between different cellular processes
93 and metabolic pathways dependent on temperature (Berrigan & Hoang, 1999; Montooth et al.
94 2003; Brown et al. 2004; Matoo et al. 2019). Then, selection under heat stress should involve
95 evolutionary changes not only in metabolic rate but also in the underlying metabolic pathways.
96 For example, several genes associated with the glucose-6-phosphate (G6P) branchpoint, an
97 important pathway in the storage and use of molecules such as glucose, glycogen, triglycerides,
98 and ATP, have shown clinal variation in their allelic frequencies along latitude (Oakeshott et al
99 1983; Duvernell & Eanes, 2000; Verrelli & Eanes, 2001; Rank & Dahlhoff, 2002, Sezgin et al.
100 2004). These findings suggest that the functional and structural properties of the enzymes
101 associated with energy metabolism are under selection, likely driven by environmental
102 temperature. Additionally, genetic variation in the G6P branchpoint results in variability in the
103 metabolic flux in ectotherms (Verrelli & Eanes, 2001), facilitating evolutionary responses among
104 fitness-related traits under stressful conditions (Zera & Zhao, 2003). Evidence from a laboratory
105 selection experiment suggests that the evolution under warm conditions results in a down-
106 regulation of metabolic pathways and an increase in fecundity in *D. simulans* (Mallard et al.
107 2018). Thus, adaptation to warming environments should involve metabolic readjustments to
108 reduce negative effects on fitness-related traits such as reproduction and development traits
109 (Sales et al., 2018; Parratt et al., 2021; García-Robledo & Baer, 2021).

110 In this work, we evaluated the evolutionary response of energy metabolism and life-
111 history traits to artificial selection for increasing heat tolerance in *D. subobscura*, using two
112 different thermal selection protocols for heat tolerance: slow and fast ramping protocols.
113 According to the assumption that individuals with low metabolic rates can withstand longer heat
114 stress, we hypothesized that increased heat tolerance evolves associated with a reduction of
115 energy metabolism, especially if the thermal selection is performed using a long (slow-ramping
116 rate) versus short (fast-ramping rate) protocols to estimate heat tolerance. Additionally, we
117 proposed that individuals with low metabolic rates will have an energy surplus, which should
118 have consequences on fitness-related traits (e.g., high developmental and reproductive success).
119 To evaluate these hypotheses, we measured the routine metabolic rate (RMR) and the activity of
120 enzymes associated with the G6P branchpoint as proxies of the energy metabolism in evolved
121 populations of *D. subobscura* previously selected for increasing heat tolerance (Mesas et al.
122 2021). We measured the activity of these enzymes in flies exposed to two different thermal
123 conditions (non-stress: 21 ºC, and stress: 32 ºC) to evaluate the thermal plasticity of G6P-related
124 enzymes. Additionally, we evaluated the early fecundity and egg-adult viability in the control
125 and selected populations to determine the effects of heat tolerance evolution on the life-history
126 traits of *D. subobscura*.
127

128 **Materials and Methods**

129 ***Heat knockdown temperature selection***

130 A mass-bred population of *D. subobscura* was established from the offspring of 100 isofemale
131 lines derived from females collected in Valdivia, Chile (39.8 ºS 73.2 ºW). Specifically, we
132 collected inseminated females using banana-yeast traps and these females were individually

133 placed into vials with David's killed-yeast *Drosophila* medium (hereafter *Drosophila* medium)
134 to establish isofemale lines. At the next generation, 10 females and 10 males from each one of
135 the 100 isofemale lines were transferred to an acrylic cage (27 x 21 x 16 cm^3) to set up one large
136 outbred population (>1500 breeding adults). In the next generation, eggs were collected and
137 transferred to 150 ml bottles with fly food at a density of 150 eggs/bottle. The bottles were
138 divided into 3 groups (15 bottles/group) and the flies that emerged from each group were
139 transferred to one acrylic cage, resulting in a total of three population cages: R1, R2, and R3.
140 After three generations, when the population cages reached a large population size and
141 environmental effects were removed, each replicated cage (R1, R2, and R3) was divided into
142 four population cages. Thus, our experimental design had 12 population cages with more than
143 1000 individuals each, which were assigned to four different artificial selection protocols in
144 triplicate: fast-ramping selection, fast-ramping control, slow-ramping selection, and slow-
145 ramping control lines (Fig. S1). During all generations, the population cages were maintained at
146 21 ºC (12:12 light:dark cycle) and fed with *Drosophila* medium in Petri dishes before the
147 artificial selection experiment.
148 For each replicate line, we randomly took 160 four-day-old virgin females, which were
149 individually placed in a vial *Drosophila* medium together with two males from the same
150 replicate line. After two days of mating, males were discarded from each vial and the presence of
151 eggs or first-instar larvae was checked. Of these 160 mated females, 120 females were randomly
152 chosen to measure their knockdown temperature and the remaining females were discarded.
153 These 120 females were individually placed in a capped 5-mL glass vial, which was attached to
154 two racks, each one with a capacity to attach 60-capped vials. Racks were immersed in a water
155 tank with an initial temperature of 28 ºC, which was controlled by a heating unit (Model ED,

156 Julabo Labortechnik, Seelbach, Germany). After an equilibration time of 10 min, the temperature
157 was increased at a rate of 0.08 ºC min^{-1} for the slow-ramping selection protocol, or 0.4 ºC min^{-1}
158 for the fast-ramping selection protocol. Thermal assays ends when the temperature reached 45
159 ºC, the temperature at which all females collapsed. Each assay was video recorded with a high-
160 resolution camera (D5100, Nikon, Tokyo, Japan). Videos were visualized to score the
161 knockdown temperature for each female fly, which was defined as the temperature at which each
162 fly ceased to move. For the selection, we selected vials containing the offspring of the 40 most
163 tolerant flies (upper 30% of each assay) to found the next generation (offspring was never
164 exposed to heat stress). Specifically, from the offspring of the selected females (40) we collected
165 four virgin females to re-established the original number of 160 females. For the fast-control and
166 slow-control lines, we measured the heat tolerance of 40 females as mentioned above and
167 randomly select the offspring of 10 of them to found the next generation.
168 Artificial selection for heat tolerance was performed for 16 generations, after which flies
169 were dumped in acrylic cages and maintained without selection for heat tolerance at 21ºC (12:12
170 light-dark cycle) until the measurement of metabolic and life-history traits. For logistic reasons,
171 we used the fast-ramping control experimental line as the control line for statistical comparisons.
172
173 ***Metabolic rate***
174 The routine metabolic rate (RMR) was measured as the amount of CO_2 produced by individuals
175 four-day-old virgin females from generation 26. RMR was measured in 15 females from each
176 replicate line, reaching a total of 135 flies (3 thermal selection protocols x 3 replicate lines x 15
177 flies). Before metabolic measurements, flies were ice-cold anesthetized and weighted using a
178 microbalance (MXA5, Radwag, Czech Republic). Then, flies were individually placed in a

179 metabolic chamber, which was made of 2 cm of a Bev-A-Line tube. The metabolic chambers
180 were connected to an eight-channel multiplexer system (RM8, Sable Systems International, NV,
181 USA) and a 5 mm^2 fabric mesh was placed between the connectors to avoid flies passing from
182 the metabolic chambers to the respirometry system. This system included seven chambers
183 containing individual flies and one empty chamber used for baseline. Metabolic chambers were
184 placed in dark conditions within a thermal cabinet (PC-1, Sable Systems International, NV,
185 USA) at a temperature of 21 ºC, which was controlled by a temperature controller (PELT-5,
186 Sable Systems International, NV, USA). CO2-free air was pumped into chambers at a flow of 15
187 mL/min (Sierra MFC-2, Sable Systems International, NV, USA) and after 30 min of
188 equilibration, CO2 production was sequentially measured in each chamber for 15 min using an
189 infrared CO_2 analyzer with a resolution of 1 ppm (Li-6251, LI-COR Bioscience, NE, USA).
190 Measurements of CO_2 were recorded, transformed to metabolic rate (µl CO_2/h), and analyzed
191 using the software Expedata (Sable Systems International, NV, USA).
192
193 ***Enzyme activity***
194 The catabolic activities of enzymes from the G6P branchpoint were measured in four-day-old
195 females from generation 27 through the appearance of NADPH in kinetic assays (Montooth et
196 al., 2003). We evaluated the activity of hexokinase (HEX: E.C. 2.7.1.1) involved in glucose
197 metabolism, phosphoglucomutase (PGM; E.C. 5.4.2.2) involved in glycogen metabolism,
198 phosphoglucose isomerase (PGI; E.C. 5.3.1.9) involved in ATP metabolism; and glucose-6-
199 phosphate dehydrogenase (G6PD; E.C. 1.1.1.49) involved in lipids metabolism.
200 For each replicate line, 100 females were collected from each population cage and pooled
201 in groups of 10 flies each. We measured the enzyme activity of the G6P branchpoint in flies

202 exposed to two thermal conditions: thermal-stressed flies (five replicates were exposed to 32 ºC
203 per 1 h and then, flies were allowed to recover at 21 ºC for 2 h); and non-stressed flies (five
204 replicates were maintained at 21 ºC for 3 h). Then, flies were quickly frozen in liquid nitrogen,
205 homogenized in 1 ml of homogenization buffer (0.01 M KH_2PO_4, 179 1.0 mM EDTA, pH 7.4),
206 and centrifuged at 2,000 rpm for 2 min at 4 ºC. The supernatant was aliquoted and maintained at
207 -80 ºC until enzymatic assays. The buffers for each assayed enzyme were as follow. ***HEX:*** 20
208 mM TrisHCl, 0.5 mM NADP, 0.2 mM $MgCl_2$, 0.36 mM ATP, 5 mM D-glucose, 0.23 units/mL
209 G6PD, pH 8.0. ***PGI:*** 20 mM TrisHCl, 0.28 mM NADP, 3 mM fructose-6-phosphate, 1.37
210 units/mL G6PD, pH 8.0. ***PGM:*** 20 mM TrisHCl, 0.5 mM NADP, 1 mM $MgCl_2$, 0.83 mM
211 glucose-1-phosphate, 3.1 units/mL G6PD, pH 8.0. ***G6PD:*** 20 mM TrisHCl, 0.2 mM NADP, 18.8
212 mM $MgCl_2$, 3.5 mM glucose-6-phosphate, pH 8.0. Enzymatic assays were performed by adding
213 25 µL extracts of flies with a protein concentration of 3500 µg/mL and 200 µL of respectively
214 assay buffer in ultraviolet plates with flat bottom wells. Immediately, the optical density (OD) to
215 340 nm was measured with multiple reads per well in a microplate reader (Infinite 200 Pro,
216 Tecan) previously heated to 29 ºC. The OD was measured each 25 s during 10 min to HEX and
217 PGI, every 10 s during 10 minutes to PGM, and every 45 s during 15 min to G6PD. Enzymatic
218 activities were estimated in triplicated and mean OD was analyzed. Blank wells containing
219 buffer assays and double-distilled water (instead of fly extracts) were measured to establish the
220 basal optical density of each reaction. Finally, the enzymatic activity was calculated as the
221 change in optical density over time.
222
223 ***Early fecundity and egg-to-adult viability***

224 Early fecundity was measured in females from generation 26. For each replicate line, we
225 collected 10 virgin females, which were individually placed in vials with *Drosophila* medium
226 together with two unrelated males from the same replicate line. After 24 h of mating, females
227 were transferred to new vials and the number of oviposited eggs in each vial was daily counted
228 using a stereomicroscope. This procedure was repeated every 24 h for eight days to obtain the
229 accumulated fecundity for each female. Additionally, we measured the egg-adult viability as
230 follows. Vials containing eggs oviposited by 5-day-old females were retained because *D.*
231 *subobscur*a reaches its peak fecundity between the age of 3 and 7 days (Foucaud et al., 2016).
232 For each vial, 20 eggs were randomly transferred to a new vial and the number of emerging
233 adults was counted.
234
235 ***Statistical analysis***
236 Normality and homoscedasticity were tested for all variables, and only the enzyme activity of
237 HEX was squared-root transformed to meet the parametric assumptions. Then, a mixed linear
238 model (hereafter 'full model') was used to evaluate the effects of thermal selection (fixed effect)
239 and replicate lines nested within thermal selection (random effect) on heat knockdown
240 temperature (eq. 1), body mass (eq. 2) and RMR (including body mass as covariate) (eq. 3):
241

242 $$\text{Knockdown temperature}_{ijk} = \mu + \text{Selection}_i + \text{Replicate(Selection)}_{ij} + e_{ijk} \quad \text{(eq. 1)}$$

243 $$\text{Body mass}_{ijk} = \mu + \text{Selection}_i + \text{Replicate(Selection)}_{ij} + e_{ijk} \quad \text{(eq. 2)}$$

244 $$\text{RMR}_{ijk} = \mu + \text{Selection}_i * \text{Body mass}_{ijk} + \text{Replicate(Selection)}_{ij} + e_{ijk} \quad \text{(eq. 3)}$$

245

For the enzyme activities, the full model included the interaction between the thermal exposure and thermal selection as fixed effects (eq. 4):

$$\text{Activity}_{ijkl} = \mu + \text{Selection}_i * \text{Exposure}_j + \text{Replicate(Selection)}_{ik} + e_{ijkl} \quad \text{(eq. 4)}$$

Early fecundity was analyzed using a generalized linear model with a Poisson (link = log) distribution with thermal selection, oviposition days and their interaction were considered as fixed effects, while replicate lines nested within thermal selection were considered as a random effect (eq. 5). As fecundity was measured for each female along 8 days, females' identity (ID) was included in the model to accomplish for the repeated-measures design:

$$\text{Fecundity}_{ijkl} = \mu + \text{Selection}_i * \text{Day}_j + \text{Replicate(Selection)}_{ik} + \text{ID}_l + e_{ijkl} \quad \text{(eq. 5)}$$

Finally, egg-to-adult viability was estimated as the proportion of emerging flies per vial, considering that 20 eggs were placed in a vial. This proportion was analyzed using a generalized linear model with a binomial distribution with thermal selection as fixed effect, and replicate lines nested within thermal selection were considered as a random effect (eq. 6):

$$\text{Viability}_{ijk} = \mu + \text{Selection}_i + \text{Replicate(Selection)}_{ij} + e_{ijk} \quad \text{(eq. 6)}$$

All statistical analysis was performed in R (R Core Team, 2021) using the function *lmer* and *glmer* of the *lme4* package for the generalized mixed model (Bates et al. 2014). Post-hoc

268 analyses were performed using a Tukey-adjusted method using the *emmeans* package (Lenth,
269 2021).
270
271 **Results**
272 ***Knockdown temperature evolution***
273 To test the evolutionary response of knockdown temperature to selection for higher thermal
274 tolerance, we compared the knockdown temperature for control and selected lines in the
275 generation 25. This comparison was independently performed for each thermal selection protocol
276 because thermal tolerance is always higher in fast ramping assays estimate than in slow ramping
277 assays (Rezende et al. 2011; Castañeda et al. 2015). We found that fast-ramping selected lines
278 evolved for significantly higher knockdown temperatures than fast-ramping control lines
279 ($mean_{fast\text{-}ramping} \pm SD = 38.59 \pm 0.98$ ºC and $mean_{fast\text{-}control} \pm SD = 37.61 \pm 1.45$ ºC; $F_{1,4} = 56.4$, $P =$
280 0.002). Similarly, slow-ramping selected lines evolved significantly higher knockdown
281 temperatures than slow-ramping control lines ($mean_{slow\text{-}ramping} \pm SD = 35.92 \pm 0.67$ ºC and
282 $mean_{slow\text{-}control} \pm SD = 35.14 \pm 0.81$ ºC; $F_{1,4} = 83.5$, $P = 0.001$). These results show that
283 differences in heat tolerance between selected and control lines were maintained after nine
284 generations without selection for heat tolerance (Mesas et al. 2021)
285
286 ***RMR and body mass***
287 Body mass was not significantly different between the control and selected lines ($\chi^2_2 = 0.46$, $P =$
288 0.794, Fig. 1A) and it did not differ between replicate lines ($\chi^2_1 = 0.03$, $P = 0.862$). On the other
289 hand, as expected, RMR and body mass were significantly correlated ($r = 0.29$, $t_{132} = 3.48$, $P =$
290 0.0007, Fig. 1B). Then, body mass was included as covariate in the mixed linear model to test

291 the effects on RMR, which showed that RMR did not differ between thermal selection regimens

292 ($\chi^2_2 = 0.28$, $P = 0.870$; Fig. 1C) and neither between the replicate lines ($\chi^2_1 = 0$, $P = 1$).

293

294 ***Enzyme activity***

295 For HEX activity (Fig. 2A), we found a significant effect of the thermal selection regimens (χ^2_2

296 $= 9.32$, $P = 0.009$), a significant decrease of HEX activity after heat stress ($\chi^2_1 = 4.46$, $P =$

297 0.035), and a non-significant interaction between thermal selection and thermal exposure ($\chi^2_2 =$

298 2.28, $P = 0.320$). Looking for selection effects on each exposure treatment (Fig. 2A), we found

299 that HEX basal activity evolved to lower values in the slow-ramping selected lines (Tukey *P*-

300 adjusted value = 0.02), whereas fast-ramping selected lines showed similar HEX basal activity as

301 control lines (Tukey *P*-adjusted value = 0.35). On the other hand, the induced activity of HEX

302 was not different between thermal selection lines (Tukey *P*-adjusted value > 0.5). For PGI

303 activity (Fig. 2B), we did not find significant effects of thermal selection ($\chi^2_2 = 3.43$, $P = 0.180$),

304 thermal exposure ($\chi^2_1 = 0.42$, $P = 0.517$), neither significant interaction between these factors

305 ($\chi^2_2 = 2.10$, $P = 0.349$). For PGM activity (Fig. 2C), we did not find significant effect of the

306 thermal selection regimens ($\chi^2_2 = 0.70$, $P = 0.706$), thermal exposure ($\chi^2_1 = 1.42$, $P = 0.233$),

307 neither significant interaction between these factors ($\chi^2_2 = 1.16$, $P = 0.560$). Finally, G6PD

308 activity was significantly different between the thermal selection regimens ($\chi^2_2 = 6.7$, $P = 0.035$;

309 Fig. 2D). On the other hand, G6PD activity did not show significant differences between thermal

310 exposure ($\chi^2_1 = 1.35$, $P = 0.245$) neither a significant interaction between thermal selection and

311 thermal exposure ($\chi^2_2 = 1.78$, $P = 0.411$). Looking for selection effects on each exposure

312 treatment, we did not find *a posteriori* significant differences between thermal selection

313 regimens within each thermal stress treatment (Tukey *P*-adjusted value > 0.05), probably

314 because differences between thermal selection regimens were close to the significance threshold

315 ($\alpha = 0.05$).

316 Regarding the variability present among replicate lines, we found that the replicate lines

317 showed a non-significant effect on the activity of all studied enzymes ($\chi^2_1 = 0$, $P = 1$).

318

319 ***Early fecundity and egg-to-adult viability***

320 Cumulative fecundity (Fig. 3) showed a significant effect of thermal selection ($\chi^2_2 = 17.83$, $P =$

321 0.0001), oviposition day ($\chi^2_1 = 9248.33$, $P < 2.2 \times 10^{-16}$), and a significant interaction between

322 both factors ($\chi^2_2 = 30.51$, $P = 2.4 \times 10^{-7}$). Specifically, the cumulative fecundity in the control

323 lines was lower than the slow-ramping selected lines (Tukey *P*-adjusted value = 0.02) and the

324 fast-ramping selected (Tukey *P*-adjusted value = 0.04). Whereas lines from both selection

325 protocols showed similar cumulative fecundity (Tukey *P*-adjusted value = 0.98). Looking for

326 selection effects on each day (Fig. 3), we observed that females of the selected lines lay more

327 eggs than females of the control lines from day 3 after mating. Additionally, cumulative

328 fecundity was not different between replicate lines ($\chi^2_1 = 2.03$, $P = 0.15$).

329 For egg-to-adult viability, we found significant effects of thermal selection ($\chi^2_2 = 16.24$,

330 $P = 0.0003$; Fig. 4). Specifically, slow-ramping selected lines showed a significantly higher egg-

331 to-adult viability than control lines (Tukey *P*-adjusted value = 0.0002), but not significantly

332 different than fast-ramping selected lines (Bonferroni *P*-adjusted value = 0.07). On the other

333 hand, fast-ramping selected lines showed similar egg-to-adult viability compared to control lines

334 (Bonferroni *P*-adjusted value = 0.17). Finally, egg-to-adult viability was significantly different

335 among replicate lines ($\chi^2_4 = 3.18$, $P = 1.4 \times 10^{-6}$), but this among-replicates variability did not
336 blur the effects of thermal selection on the egg-to-adult viability.
337
338 **Discussion**
339 The mechanisms of tolerance to environmental stress are fundamental for the persistence of
340 natural populations and biological diversity. Temperature is a conspicuous environmental
341 variable that influences the evolution of metabolic and life-history traits in ectotherms (Gilloly et
342 al. 2001; Addo-Bediako et al. 2002; Brown et al. 2004; Padfield et al. 2016, Mallard et al. 2018).
343 In this work, we found that the experimental populations that were selected for an increasing
344 thermal tolerance showed changes in enzyme activities related to energy metabolism, and also
345 evolutionary responses of fitness-related traits in *D. subobscura*. However, heat tolerance
346 evolution was not associated with changes in the metabolic rate in selected populations.
347 Despite the ubiquitous effects of temperature on metabolism, our findings show no
348 evidence of metabolic depression associated with heat tolerance evolution in *D. subobscura*. To
349 explain this finding, we must recognize that there is contrasting evidence about the temperature
350 effects on the evolution of metabolism. For instance, different studies have reported that species
351 living in temperate environments have lower metabolic rates than species inhabiting cold habitats
352 (Addo-Bediako et al. 2002; Sylvestre et al. 2007; Schaefer & Walters, 2010; Sinnatamby et al.
353 2015). While several studies have found a reduction in metabolic rate in response to warm
354 conditions (Padfield et al. 2016; Mallard et al. 2017; Pilakouta et al. 2020). On the other hand,
355 and in agreement with our findings, there is evidence that does not support the effects of
356 temperature on the evolution of metabolism at intra- (Alton et al. 2017) and interspecific level
357 (Messamah et al. 2017). A plausible explanation is that the evolution of metabolic rate could

358 depend on the interaction between temperature and resource availability, which would difficult
359 the detection of metabolic changes under non-stressful maintenance conditions (Alton et al.
360 2017). In this way, our control and evolved populations were fed 'ad libitum' before measures,
361 which could hide correlated responses of metabolic rate to heat tolerance evolution. Additionally,
362 metabolic rate was only measured at a single temperature (21ºC), which does not allow us to test
363 the effect of heat tolerance evolution on the thermal sensitivity of metabolic rate (e.g., Q_{10}). If
364 this is true, it is expected that differences in metabolic rate between selected and control lines
365 would have been larger at higher temperatures (Colinet et al. 2015).

366 Another explanation for our findings is that metabolic rate is a complex trait that represents
367 the total flux of energy at an organismic level and it does not necessarily account for changes in
368 enzymes related to energy metabolism (O'Brien & Suarez, 2001). For branching pathways as the
369 G6P-branchpoint, there is evidence that selection acts on enzymes capable of controlling the flux
370 allocation in *Drosophila* species (Flowers et al., 2007). Here, we found evidence that artificial
371 selection for increasing heat tolerance changes the activity of the HEX and G6PD enzymes: HEX
372 evolved to lower enzyme activity in the slow-ramping selected lines, but this effect was only
373 detected when flies were exposed to non-stressful thermal conditions (21ºC); whereas the G6PD
374 activity showed significant differences between thermal selection regimens, but the *a posteriori*
375 analysis did not show differences between evolved and control lines (likely because of the *P*-
376 value was close to the significance threshold). These changes in the activity of the G6P
377 branchpoint enzymes agree with a study that found that populations evolving in thermally
378 fluctuating environments of *D. simulans* showed a reduction in the gene expression of enzymes
379 participating in energy processing (Mallard et al. 2018). Particularly, we think that the lower
380 enzyme activity could be an evolutionary consequence to heat tolerance evolution in slow-

381 ramping selected lines because flies with high heat tolerance should have had a low metabolism
382 to withstand heat stress for a longer time (Santos et al. 2012).
383 Our results for the fitness-related traits also provide indirect evidence for the adaptive
384 value of the energy-saving strategies (e.g., metabolic depression). Several studies have found
385 associations between metabolic enzymes and reproductive traits in insects (Kageyama &
386 Ohnishi, 1971; Clark & Fucito, 1998; Harshman et al. 1999; Mallard et al. 2018). We propose
387 that this association can be explained by the pace-to-life syndrome, which proposes that
388 individuals with a fast metabolism should grow faster than their counterparts with a slow
389 metabolism, but at the cost to have a shorter lifespan and lower fecundity (Stearns 1989;
390 Polverino et al. 2018; Tüzün et al. 2022). However, our findings only match with the pace-to-life
391 syndrome because a reduced metabolic activity was found only for the slow-ramping selected
392 lines. Whereas an explanation for the higher fecundity exhibited by the fast-ramping selected
393 lines is unclear, despite we can observe that fast-ramping selected lines exhibited a lower activity
394 of G6PD than control lines (but this difference was not significantly different between both
395 groups). Regarding egg-to-adult viability, slow-ramping selected lines exhibited viability 1.5
396 times higher than control lines with several vials reaching up to 100% viability, whereas fast-
397 ramping selected lines did not differ from control lines. Previous results have found that low
398 activities of HEX and G6PD were associated with short development times in *D. melanogaster*
399 and *D. subobcura* (Marinkovié et al. 1986), whereas *D. melanogaster* flies that exhibited low
400 activity of HEX (as well as other metabolic enzymes) showed longer lifespan (Talbert et al.,
401 2015).
402 In conclusion, heat tolerance evolution has positive consequences on fitness-related traits,
403 including increased fecundity and preadult survival. Despite some evidence showing that thermal

limits have limited evolutionary potential (Kellermann et al. 2012; Kelly et al. 2012), we have found through several works that upper thermal limits have enough genetic variation to respond to selection (Castañeda et al. 2019), which allows to *D. subobscura* populations adapt to local conditions (Castañeda et al. 2015) and respond to selection (Mesas et al. 2021; but see Santos et al. 2022 for no evolutionary response to warm temperatures). Thus, heat tolerance can evolve under different thermal scenarios but with different outcomes on associated traits depending on the intensity of thermal stress. Therefore, spatial and temporal variability of thermal stress intensity should be taken into account in future studies (see Buckley et al., 2013; Rezende et al. 2020) if we want to understand and predict the adaptive response to ongoing and future climatic conditions.

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Conflict of interest disclosure

The authors declare they have no conflict of interest relating to the content of this article.

Author contributions

A.M. designed experiments, conducted experiments and statistical analyses and wrote the first draft of the manuscript. L.E.C. conceived the original idea, designed experiments, conducted statistical analyses, provided funds for all experiments, and edited and wrote the manuscript.

Data, script and code availability

Data and script are available in https://doi.org/10.6084/m9.figshare.20373180.v1

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Supplementary Information

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References

Addo-Bediako, A., Chown, S. L., & Gaston, K. J. (2002). Metabolic cold adaptation in insects: a large-scale perspective. Functional Ecology, 16(3), 332-338. https://doi.org/10.1046/j.1365-2435.2002.00634.x

Alton, L. A., Condon, C., White, C. R., & Angilletta Jr, M. J. (2017). Colder environments did not select for a faster metabolism during experimental evolution of *Drosophila melanogaster*. Evolution, 71(1), 145-152. https://doi.org/10.1111/evo.13094

Angilletta Jr, M. J.(2009). Thermal Adaptation: a Theoretical and Empirical Synthesis. Oxford, UK: Oxford University Press.

449 Artacho, P. & Nespolo, R.F. (2009). Natural selection reduces energy metabolism in the garden
450 snail, *Helix aspersa* (*Cornu Aspersum*). Evolution, 63, 1044-1050.
451 https://doi.org/10.1111/j.1558-5646.2008.00603.x
452 Atkinson D.E. (1977). Cellular Energy Metabolism and Its Regulation. Academic Press. New
453 York.
454 Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models
455 using lme4. arXiv preprint arXiv:1406.5823
456 Berrigan, D., & Hoang, A. (1999). Correlation between enzyme activities and routine metabolic
457 rate in *Drosophila*. Journal of Evolutionary Biology, 12(2), 258-262.
458 https://doi.org/10.1046/j.1420-9101.1999.00027.x
459 Brown, J. H., Gillooly, J. F., Allen, A. P., Savage, V. M., & West, G. B. (2004). Toward a
460 metabolic theory of ecology. Ecology, 85(7), 1771-1789. https://doi.org/10.1890/03-9000
461 Brown, E. B., Slocumb, M. E., Szuperak, M., Kerbs, A., Gibbs, A. G., Kayser, M. S., & Keene,
462 A. C. (2019). Starvation resistance is associated with developmentally specified changes in
463 sleep, feeding and metabolic rate. Journal of Experimental Biology, 222(3), jeb191049.
464 https://doi.org/10.1242/jeb.191049
465 Buckley, L. B., Miller, E. F., & Kingsolver, J. G. (2013). Ectotherm thermal stress and
466 specialization across altitude and latitude. Integrative and Comparative Biology, 53(4),
467 571-581. https://doi.org/10.1093/icb/ict026
468 Castañeda, L. E., Rezende, E. L., & Santos, M. (2015). Heat tolerance in *Drosophila subobscura*
469 along a latitudinal gradient: Contrasting patterns between plastic and genetic responses.
470 Evolution, 69, 2721-2734. https://doi.org/10.1111/evo.12757

Castañeda, L.E., Romero-Soriano, V., Mesas, A., Roff, D. A., & Santos, M. (2019). Evolutionary potential of thermal preference and heat tolerance in *Drosophila subobscura*. Journal of Evolutionary Biology, 32(8), 818-824. https://doi.org/10.1111/jeb.13483

Chen, I. C., Hill, J. K., Ohlemüller, R., Roy, D. B., & Thomas, C. D. (2011). Rapid range shifts of species associated with high levels of climate warming. Science, 333(6045), 1024-1026. https://doi.org/doi/10.1126/science.1206432

Chick, L. D., Waters, J. S., & Diamond, S. E. (2021). Pedal to the metal: Cities power evolutionary divergence by accelerating metabolic rate and locomotor performance. Evolutionary applications, 14(1), 36-52. https://doi.org/10.1111/eva.13083

Clark, A. G., & Fucito, C. D. (1998). Stress tolerance and metabolic response to stress in *Drosophila melanogaster*. Heredity, 81(5), 514-527. https://doi.org/10.1046/j.1365-2540.1998.00414.x

Colinet, H., Sinclair, B. J., Vernon, P. & Renault. D. (2015). Insects in fluctuation thermal environments. Annual Review of Entomology, 60, 123-140. https://doi.org/10.1146/annurev-ento-010814-021017

Crozier, L. G., & Hutchings, J. A. (2014). Plastic and evolutionary responses to climate change in fish. Evolutionary Applications, 7(1), 68-87. https://doi.org/10.1111/eva.12135

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., & Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. Proceedings of the National Academy of Sciences, 105(18), 6668-6672. https://doi.org/10.1073/pnas.0709472105

492 Djawdan, M., Rose, M.R., & Bradley, T.J. (1997). Does selection for stress resistance lower
493 metabolic rate? Ecology, 78, 828-837. https://doi.org/10.1890/0012-
494 9658(1997)078[0828:DSFSRL]2.0.CO;2
495 Duvernell, D. D., & Eanes, W. F. (2000). Contrasting molecular population genetics of four
496 hexokinases in *Drosophila melanogaster, D. simulans* and *D. yakuba*. Genetics, 156(3),
497 1191-1201. https://doi.org/10.1093/genetics/156.3.1191
498 Flowers, J. M., Sezgin, E., Kimagai, S., Duvernell, D. D., Matzkin, L. M. Schmidt, P. S., &
499 Eanes, W. F. (2007). Adaptive evolution of metabolic pathways in *Drosophila*. Molecular
500 Biology and Evolution, 24(6), 1347-1354. https://doi.org/10.1093/molbev/msm057
501 Foucaud, J., Moreno, C., Pascual, M., Rezende, E.L., Castañeda, L.E., Gibert, P., & Mery, F.
502 (2016). Introduced *Drosophila subobscura* populations perform better than native
503 populations during oviposition choice task due to increased fecundity but similar learning
504 ability. Ecology and Evolution, 6(6), 1725-1736. https://doi.org/10.1002/ece3.2015
505 García-Robledo, C., & Baer, C.S. (2021). Positive genetic covariance and limited thermal
506 tolerance constrain tropical insect responses to global warming. Journal of Evolutionary
507 Biology, 34(9), 1432–1446. https://doi.org/10.1111/jeb.13905
508 Gaston, K.J., Chown, S.L., Calosi, P., Bernardo, J., Bilton, D.T., Clarke, A., Clusella-Trullas, S.,
509 Ghalambor, C.K., Konarzewski, M., Peck, L.S., Porter, W.P., Pörtner, H.O., Rezende,
510 E.L., Schulte, P.M., Spicer, J.I., Stillman, J.H., Terblanche, J.S., & van Kleunen, M.
511 (2009). Macrophysiology: a conceptual reunification. The American Naturalist,174(5),
512 595-612. https://doi.org/10.1086/605982

Gillooly, J. F., Brown, J. H., West, G. B., Savage, V. M., & Charnov, E. L. (2001). Effects of size and temperature on metabolic rate. Science, 293(5538), 2248-2251. https://doi.org/10.1126/science.1061967

Guppy, M., & Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biological Reviews, 74(1), 1-40. https://doi.org/10.1017/S0006323198005258

Harshman, L. G., Hoffmann, A. A., & Clark, A. G. (1999). Selection for starvation resistance in *Drosophila melanogaster*: physiological correlates, enzyme activities and multiple stress responses. Journal of Evolutionary Biology, 12(2), 370-379. https://doi.org/10.1046/j.1420-9101.1999.00024.x

Hoffmann, A. A., & Parsons, P. A. (1989). Selection for increased desiccation resistance in *Drosophila melanogaster*: additive genetic control and correlated responses for other stresses. Genetics, 122(4), 837-845. https://doi.org/10.1093/genetics/122.4.837

Huey, R. B., & Kingsolver, J. G. (1993). Evolution of resistance to high temperature in ectotherms. The American Naturalist,142, S21-S46. https://doi.org/10.1086/285521

Kageyama, T., & Ohnishi, E. (1971). Carbohydrate metabolism in the eggs of the silkworm, *Bombyx mori*. I. Absence of phosphofructokinase in the diapausing egg. Development, Growth & Differentiation, 13(2), 97-106. https://doi.org/10.1111/j.1440-169X.1971.00097.x

Kellermann, V., Loeschcke, V., Hoffmann, A.A., Kristensen, T.N., Fløjgaard, C., David, J.R., Svenning, J.C., & Overgaard, J. (2012). Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95

Drosophila species. Evolution, 66, 3377-3389. https://doi.org/10.1111/j.1558-5646.2012.01685.x

Kelly, M. W., Sanford, E., & Grosberg, R.K. (2012). Limited potential for adaptation to climate change in a broadly distributed marine crustacean. Proceedings of the Royal Society B: Biological Sciences, 279(1727), 349-356. https://doi.org/10.1098/rspb.2011.0542

Lenth, R. (2021). Estimated Marginal Means, aka Least-Squares Means. R Package version 1.3.4. https://cran.r-project.org/web/packages/emmeans/index.

Mallard, F., Nolte, V., Tobler, R., Kapun, M., & Schlötterer, C. (2018). A simple genetic basis of adaptation to a novel thermal environment result in complex metabolic rewiring in *Drosophila*. Genome Biology, 19(1), 1-15. https://doi.org/10.1186/s13059-018-1503-4

Marinković, D., Milošević, M., & Milanović, M. (1986). Enzyme activity and dynamics of *Drosophila* development. Genetica, 70(1), 43-52. https://doi.org/10.1007/BF00123213

Matoo, O.B., Julick, C. R., & Montooth, K. L. (2019). Genetic variation for ontogenetic shifts in metabolism underlies physiological homeostasis in *Drosophila*. Genetics, 212(2), 537-552. https://doi.org/10.1534/genetics.119.302052

McGaughran, A., Laver, R., & Fraser, C. (2021). Evolutionary responses to warming. Trends in Ecology & Evolution. 36, 591-600. https://doi.org/10.1016/j.tree.2021.02.014

Mesas, A., Jaramillo, A., & Castañeda, L. E. (2021). Experimental evolution on heat tolerance and thermal performance curves under contrasting thermal selection in *Drosophila subobscura*. Journal of Evolutionary Biology, 34(5), 767-778. https://doi.org/10.1111/jeb.13777

Messamah, B., Kellermann, V., Malte, H., Loeschcke, V., & Overgaard, J. (2017). Metabolic cold adaptation contributes little to the interspecific variation in metabolic rates of 65

species of Drosophilidae. Journal of Insect Physiology, 98, 309-316. https://doi.org/10.1016/j.jinsphys.2017.02.003

Montooth, K.L., Marden, J.H. & Clark, A.G. (2003). Mapping determinants of variation in energy metabolism, respiration and flight in *Drosophila*. Genetics, 165(2), 623-635. https://doi.org/10.1093/genetics/165.2.623

Oakeshott, J. G., Chambers, G. K., Gibson, J. B., Eanes, W. F., & Willcocks, D. A. (1983). Geographic variation in G-6-pd and Pgd allele frequencies in *Drosophila melanogaster*. Heredity, 50(1), 67-72.

O'Brien, D. M., & Suarez, R. K. (2001). Fuel use in hawkmoth (*Amphion floridensis*) flight muscle: enzyme activities and flux rates. Journal of Experimental Zoology, 290(2), 108-114. https://doi.org/10.1002/jez.1040

Padfield, D., Yvon-Durocher, G., Buckling, A., Jennings, S., & Yvon-Durocher, G. (2016). Rapid evolution of metabolic traits explains thermal adaptation in phytoplankton. Ecology Letters, 19(2), 133-142. https://doi.org/10.1111/ele.12545

Parratt, S.R., Walsh, B.S., Metelmann, S., White, N., Manser, A., Bretman, A.J., Hoffmann, A.A., Snook, R.R. & Price, T. A. (2021). Temperatures that sterilize males better match global species distributions than lethal temperatures. Nature Climate Change, 11(6), 481-484. https://doi.org/10.1038/s41558-021-01047-0

Pilakouta, N., Killen, S. S., Kristjánsson, B. K., Skúlason, S., Lindström, J., Metcalfe, N. B., & Parsons, K. J. (2020). Multigenerational exposure to elevated temperatures leads to a reduction in standard metabolic rate in the wild. Functional Ecology, 34(6), 1205-1214. https://doi.org/10.1111/1365-2435.13538

Polverino, G., Santostefano, F., Díaz-Gil, C., & Mehner, T. (2018). Ecological conditions drive pace-of-life syndromes by shaping relationships between life history, physiology and behaviour in two populations of Eastern mosquitofish. Scientific Reports, 8(1), 14673. https://doi.org/10.1038/s41598-018-33047-0

Pörtner, H. O., Bennett, A. F., Bozinovic, F., Clarke, A., Lardies, M. A., Lucassen, M., Pelster, B., Schiemer, F., & Stillman, J. H. (2006). Trade-offs in thermal adaptation: the need for a molecular to ecological integration. Physiological and Biochemical Zoology, 79(2), 295-313. https://doi.org/10.1086/499986

R Core Team. (2021). R: A language and environment for statistical computing (version 4.0.2). R Foundation for Statistical Computing, Vienna, Austria. Retrieved from https://www.R-project.org

Rank, N. E., & Dahlhoff, E. P. (2002). Allele frequency shifts in response to climate change and physiological consequences of allozyme variation in a montane insect. Evolution, 56(11), 2278-2289. https://doi.org/10.1111/j.0014-3820.2002.tb00151.x

Regan, M. D., Gill, I. S., & Richards, J. G. (2017). Metabolic depression and the evolution of hypoxia tolerance in threespine stickleback, *Gasterosteus aculeatus*. Biology Letters, 13(11), 20170392. https://doi.org/10.1098/rsbl.2017.0392

Rezende, E. L., Tejedo, M., & Santos, M. (2011). Estimating the adaptive potential of critical thermal limits: methodological problems and evolutionary implications. Functional Ecology, 25(1), 111-121. https://doi.org/10.1111/j.1365-2435.2010.01778.x

Rezende, E. L., Bozinovic, F., Szilágyi, A., & Santos, M. (2020). Predicting temperature mortality and selection in natural *Drosophila* populations. Science, 369(6508), 1242-1245. https://doi.org/10.1126/science.aba9287

Sales, K., Vasudeva, R., Dickinson, M.E., Godwin, J.L., Lumley, A.J., Michalczyk, Ł., Hebberecht, L., Thomas, P., Franco, A., & Gage, M. (2018). Experimental heatwaves compromise sperm function and cause transgenerational damage in a model insect. Nature Communications, 9(1), 4771. https://doi.org/10.1038/s41467-018-07273-z

Santos, M., Castañeda, L. E., & Rezende, E. L. (2012). Keeping pace with climate change: what is wrong with the evolutionary potential of upper thermal limits? Ecology and Evolution, 2(11), 2866-2880. https://doi.org/10.1002/ece3.385

Santos, M. A., Carromeu-Santos, A., Quina, A.S., Santos, M., Matos, M., & Simões, P. (2022). No evidence for short-term evolutionary response to a warming environment in drosophila. Evolution, 75(11), 2816-2829. https://doi.org/10.1111/evo.14366

Schaefer, J., & Walters, A. (2010). Metabolic cold adaptation and developmental plasticity in metabolic rates among species in the *Fundulus notatus* species complex. Functional Ecology, 24(5), 1087-1094. https://doi.org/10.1111/j.1365-2435.2010.01726.x

Sezgin, E., Duvernell, D. D., Matzkin, L. M., Duan, Y., Zhu, C. T., Verrelli, B. C., & Eanes, W. F. (2004). Single-locus latitudinal clines and their relationship to temperate adaptation in metabolic genes and derived alleles in *Drosophila melanogaster*. Genetics, 168(2), 923-931. https://doi.org/10.1534/genetics.104.027649

Sinnatamby, R.N, Brian Dempson, J., Reist, J. D., & Power, M. (2015). Latitudinal variation in growth and otolith-inferred field metabolic rates of Canadian young-of-the-year Arctic charr. Ecology of Freshwater Fish, 24(3), 478-488. https://doi.org/10.1111/eff.12166

Stearns, S.C. (1989). Trade-Offs in Life-History Evolution. Functional Ecology, 3(3), 259-268. https://doi.org/10.2307/2389364

625 Storey, K. B., & Storey, J. M. (2004). Metabolic rate depression in animals: transcriptional and
626 translational controls. Biological Reviews, 79(1), 207-233. http:// doi.org/10.1007/s00360-
627 007-0143-x
628 Sylvestre, E. L., Lapointe, D., Dutil, J. D., & Guderley, H. (2007). Thermal sensitivity of
629 metabolic rates and swimming performance in two latitudinally separated populations of
630 cod, *Gadus morhua* L. Journal of Comparative Physiology B, 177(4), 447-460.
631 https://doi.org/10.1007/s00360-007-0143-x.pdf
632 Talbert, M. E., Barnett, B., Hoff, R., Amella, M., Kuczynski, K., Lavington, E., Koury, S., Brud,
633 E., & Eanes, W. F. (2015). Genetic perturbation of key central metabolic genes extends
634 lifespan in *Drosophila* and affects response to dietary restriction. Proceedings of the Royal
635 Society B, 282(1815), 20151646. https://doi.org/10.1098/rspb.2015.1646
636 Tüzün, N. & Robby, S. (2022). A fast pace-of-life is traded off against a high thermal
637 performance. Proceedings of the Royal Society B, 289, 20212414.
638 http://doi.org/10.1098/rspb.2021.2414
639 Umina, P. A., Weeks, A. R., Kearney, M. R., McKechnie, S. W., & Hoffmann, A. A. (2005). A
640 rapid shift in a classic clinal pattern in *Drosophila* reflecting climate change. Science,308
641 (5722), 691-693. https:/doi.org/ 10.1126/science.1109523
642 Verrelli, B. C., & Eanes, W. F. (2001). The functional impact of Pgm amino acid polymorphism
643 on glycogen content in *Drosophila melanogaster*. Genetics, 159(1), 201-210.
644 https://doi.org/10.1093/genetics/159.1.201
645 Zera, A. J., & Zhao, Z. (2003). Morph-dependent fatty acid oxidation in a wing-polymorphic
646 cricket: implications for the trade-off between dispersal and reproduction. Journal of Insect
647 Physiology, 49(10), 933-943. https://doi.org/10.1016/S0022-1910(03)00152-5

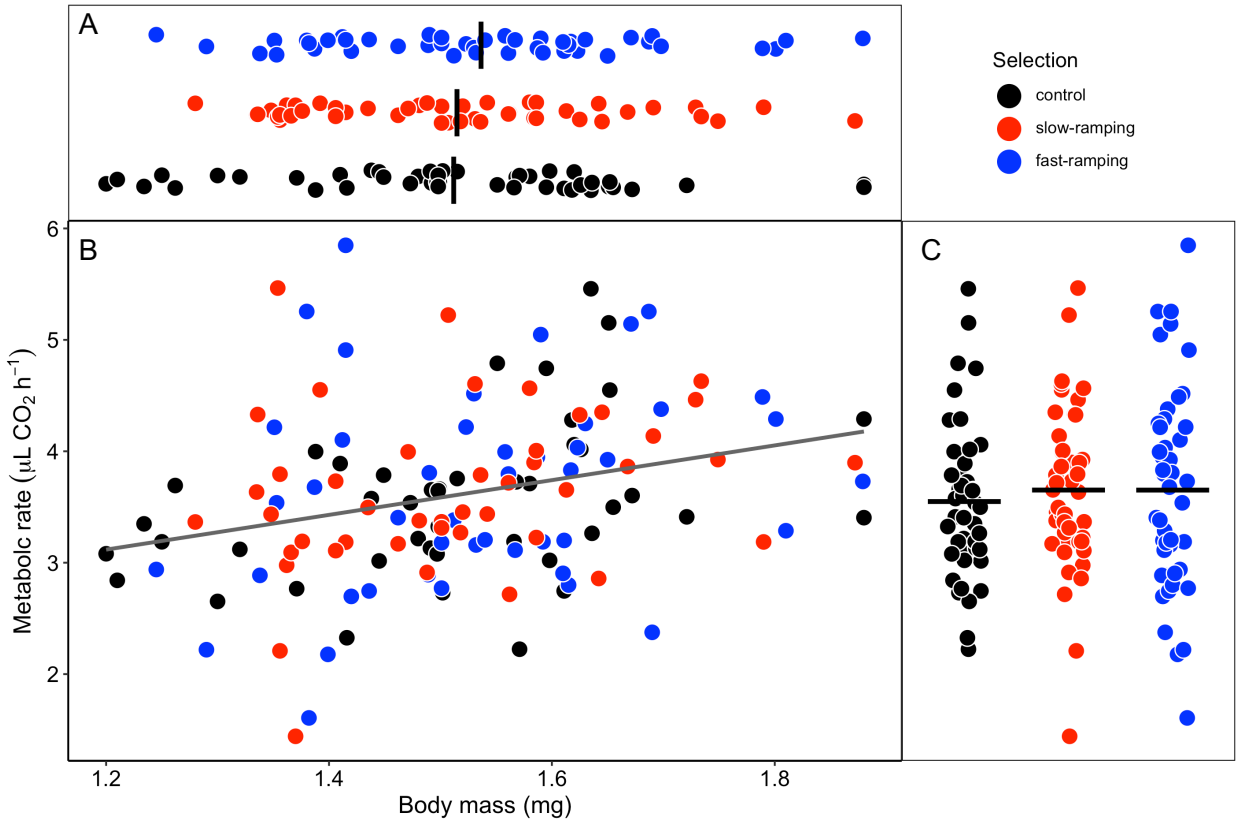


Figure 1. Body mass (A), the relationship between body mass and metabolic rate (B), and resting metabolic rate (C) for flies of control lines (black circles), slow-ramping selected lines (red circles), and fast-ramping selected lines (blue circles) for increasing heat tolerance in *Drosophila subobscura*. In panel B, the grey line represents the linear regression between both traits. Non-significant differences between thermal selection regimens were found for body mass and resting metabolic rate (see text).

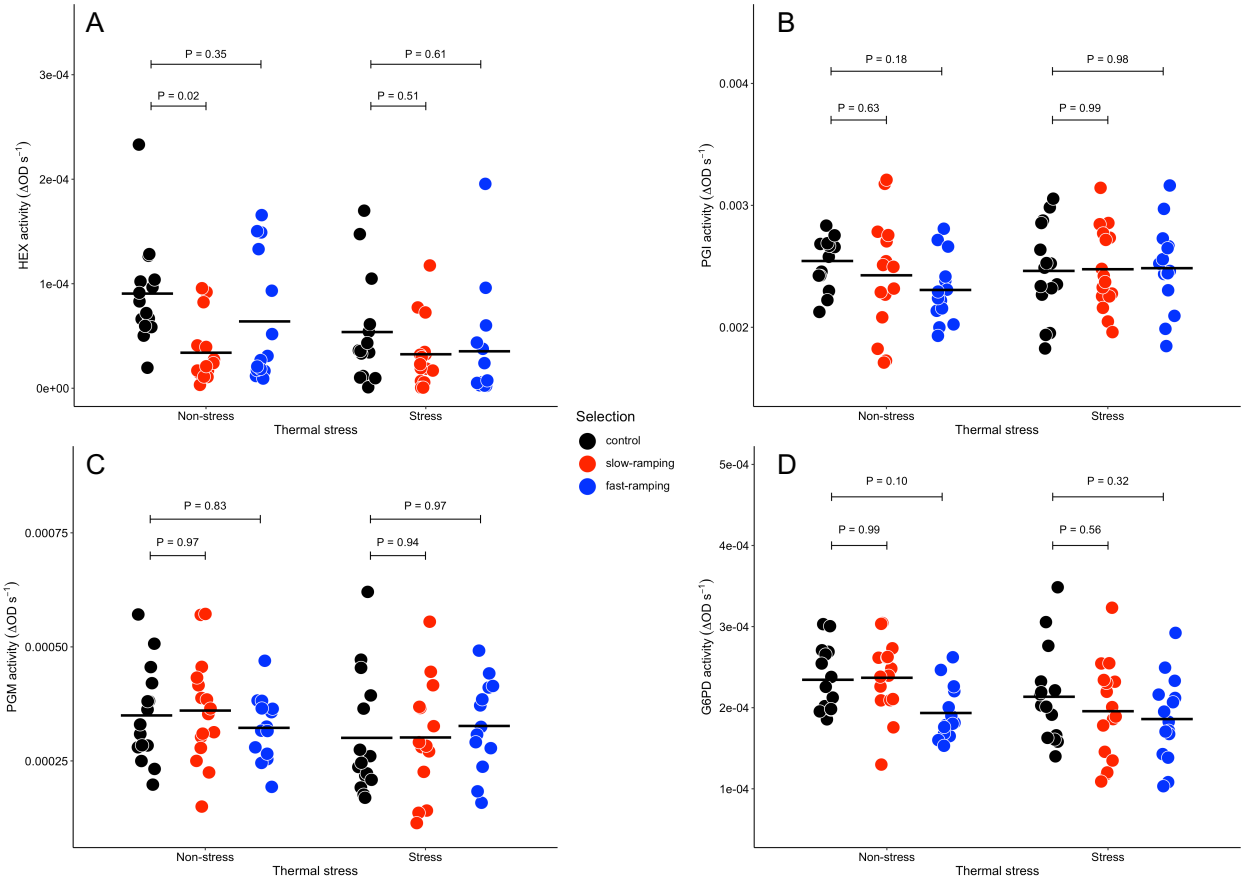


Figure 2. The activity of enzymes related to G6P branchpoint of flies of control lines (black circles), slow-ramping selected lines (red circles), and fast-ramping selected lines (blue circles) for increasing heat tolerance in *Drosophila subobscura* exposed to non-stressful (21 ºC) and stressful conditions (32 ºC): (A) hexokinase (HEX); (B) phosphoglucoisomerase (PGI); (C) phophoglucomutase (PGM); and (D) glucose-6-phosphate dehydrogenase (G6PD). Horizontal lines show the mean for each group. *P*-values above whiskers show the results of *a posteriori* comparisons between control and selected lines using Tukey tests.

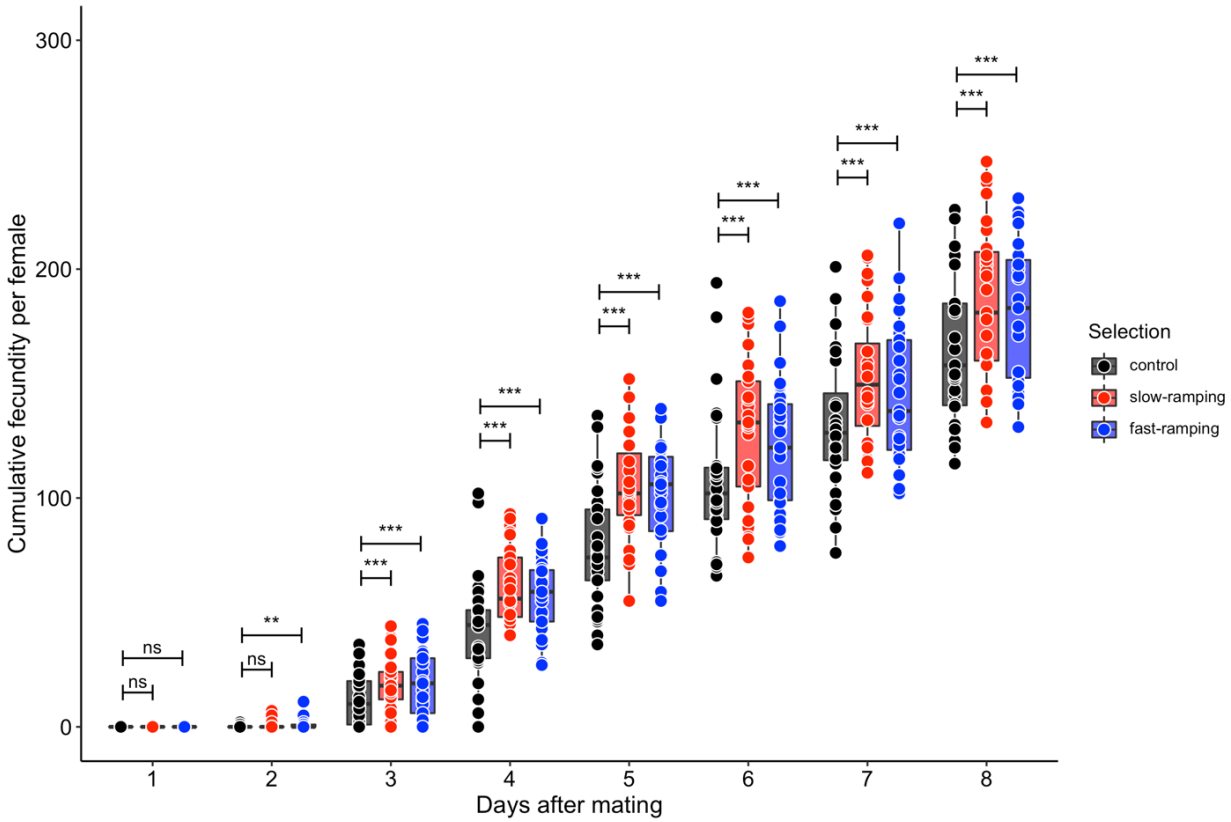
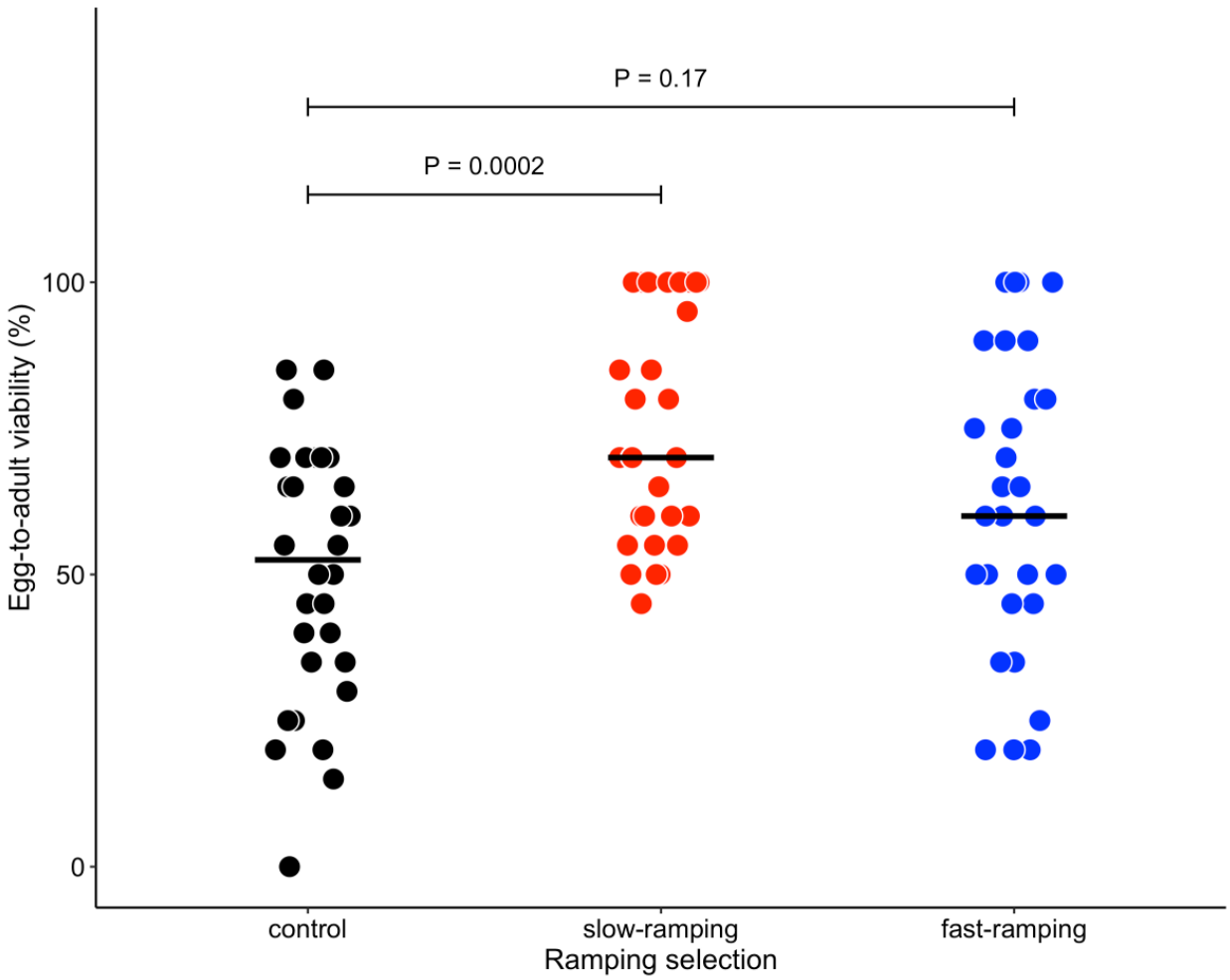


Figure 3. Cumulative fecundity during the first 8 days after mating (early fecundity) for females of control lines (black circles and boxplots), slow-ramping selected lines (red circles and boxplots), and fast-ramping selected lines (blue circles and boxplots) for increasing heat tolerance in *Drosophila subobscura*. Boxplots show the median, the interquartile range (IRQ) and vertical whiskers represent the 1.5*IQR. *P*-values above horizontal whiskers show the results of *a posteriori* comparisons between control and selected lines using Tukey tests (ns: P > 0.05, *: P < 0.05, **: P < 0.01, ***: P < 0.001).



678

679 **Figure 4**. Egg-to-adult viability of flies of control lines (black circles), slow-ramping selected

680 lines (red circles), and fast-ramping selected lines (blue circles) for increasing heat tolerance in

681 *Drosophila subobscura*. Horizontal lines show the mean for each group. P-values above

682 horizontal whiskers show the results of *a posteriori* comparisons between control and selected

683 lines using Tukey tests.